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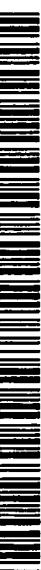
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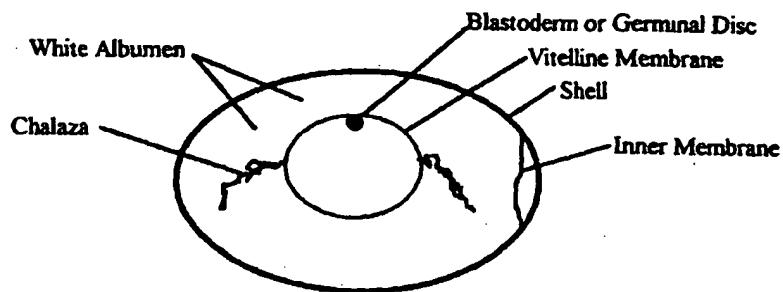
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(54) Title: METHODS FOR MANIPULATING THE AVIAN GENOME



**WO 00/75300 A2**

(57) Abstract: The invention features a method for introducing a nucleic acid molecule into the genome of an avian species by contacting *in vivo* a blastodermal cell of a fertilized egg with the nucleic acid molecule, which nucleic acid is not associated with a viral coat protein. The invention also encompasses transgenic avian animals and methods of producing such transgenic animals.

**METHODS FOR MANIPULATING THE AVIAN GENOME****BACKGROUND OF THE INVENTION**

The invention relates to transgenic avian animals.

The ability to create transgenic animals from different species has had great impact on both biomedical research and the biotechnology industry. Transgenic technology has been applied to both laboratory and domestic species for the study of human disease (see, for example, Synder, B.W., et al., Mol. Reprod. and Develop. 40:419-428 (1995)), development of improved agricultural livestock (see, for example, Ebert, K.M. et al., Animal Biotechnology 1:145-159 (1990)), production of pharmaceuticals in milk (see, for review article, Ebert, K.M. and J.P. Selgrath, "Changes in Domestic Livestock through Genetic Engineering" in Applications in Mammalian Development, Cold Spring Harbor Laboratory Press, 1991)), and xenotransplantation (see, for example, Osman, N., et al., Proc. Natl. Acad. Sci USA 94:14677-14682 (1997)). However, the basic technique of microinjection used to create many of these transgenic animals is inefficient, costly, and not easily applicable to all species such as the chicken (see, for example, Love, J., et al., BioTechnology 12:60-63 (1994)).

**SUMMARY OF THE INVENTION**

The invention features methods of manipulating genomic DNA in avian species and to generate transgenic avian animals. A method for introducing a nucleic acid molecule into the genome of an avian species is carried out by contacting *in vivo* a blastodermal cell of a fertilized hard shelled egg with the nucleic acid molecule. Preferably, the nucleic acid molecule is not associated with a viral coat protein, e.g., the nucleic acid is not delivered in a viral particle. The nucleic acid molecule is introduced directly into the germinal disc of the egg. To avoid disrupting the germinal disc (or blastoderm) or dispersing the blastodermal cells, the nucleic acid is delivered in a volume that is less than the volume of the germinal disc (approximately 100 microliters or less). For example, the volume is greater than 1 microliter and less than 0.5 milliliters and introduction of the nucleic acid does not rupture the area opaca (membrane or sheath surrounding the germinal disc or blastoderm). In preferred embodiments, the nucleic acid is delivered in a volume of 5-100 microliters, more preferably 40-60 microliters, and most preferably, 10-20 microliters. To target blastodermal cells, the nucleic acid is delivered directly into the blastoderm or germinal disc and not to an area adjacent to or below the blastoderm. The thick albumen around the germinal disc is not

removed before, during, or after the delivery process. The nucleic acid is delivered by passing a needle or microinjection pipet directly through the shell and underlying membrane. Little or no air is introduced into the egg, and the hole in the membrane left by the pipet or needle is small and self-sealing. Accordingly, the method does not require deposition of an aqueous liquid over the opening of the egg to minimize the inadvertent introduction of air into the egg and does not require sealing the opening of the egg after nucleic acid delivery. Optionally, the blastodermal cell is exposed to an electrical current *in vivo*, e.g., by applying an electrical current across the egg.

The method is useful to introduce a nucleic acid such as DNA into the genome of any avian species such as a chicken, an ostrich, an emu, a turkeys, a duck, a goose, a quail a parrot, a parakeet, a cockatoo, or a cockatiel to produce therapeutic proteins or to make an avian model for a non-avian, e.g., human, disease state. For example, the method is used to deliver DNA to blastodermal cells in a fertilized egg of any breed of chicken or any hybrid breed of chicken. Chicken breeds include White Leghorn, White Plymouth Rock, Barred Plymouth Rock, Rhode Island Red, New Hampshire and Dark Cornish.

Nucleic acid delivery is timed to optimize uptake of the nucleic acid by blastodermal (totipotent) cells and minimize (or eliminate) uptake by cells which have begun to differentiate into various tissue types. Thus, a blastodermal cell in a fertilized egg is contacted with nucleic acid during developmental stage X of the egg. Delivery takes place at a time after oviposition but before incubation of the egg (at which time cell division and cell differentiation takes place).

The nucleic acid encodes a polypeptide or antisense molecule. For example, the nucleic acid contains a sequence encoding an antibody or fragment thereof. The term antibody encompasses an intact tetrameric antibody (e.g., a monoclonal antibody) as well as an immunologically active antibody fragment, e.g., a Fab or (Fab)<sub>2</sub> fragment, an engineered single chain F<sub>v</sub> molecule, a chimeric molecule (e.g., an antibody which contains the binding specificity of one antibody, e.g., of murine origin, and the remaining portions of another antibody, e.g., of human origin. Preferably, the antibody or fragment thereof is of human origin. Other polypeptides include an insulin polypeptide (such as a human or porcine insulin polypeptide), a growth hormone polypeptide, a calcitonin polypeptide, or a serum albumin polypeptide. For example, the transgenic nucleic acid encodes a porcine single chain insulin polypeptide.

To direct transcription of the transgenic nucleic acid in blastodermal cells of an egg, the nucleic acid construct contains an egg-specific transcriptional regulatory element. Transcriptional regulatory sequence is a generic term used throughout the specification to refer to DNA sequences, such as initiation signals, enhancers, and promoters, which induce or control transcription of protein coding sequences to which they are operably linked.

5 Transcription of the recombinant gene or transgene is under the control of a promoter sequence as well as other transcriptional regulatory sequences. For example, an ovalbumin promoter sequence is used to direct expression of a transgene and a regulatory sequences derived from a chicken lysozyme gene is used to direct equal or equivalent expression of both chains of a 10 transgenic tetrameric antibody molecule. By "promoter" is meant a minimal DNA sequence sufficient to direct transcription. Promoters may be constitutive or inducible, and may be coupled to other regulatory sequences or "elements" which render promoter-dependent gene 15 expression cell-type specific, tissue-specific or inducible by external signals or agents; such elements may be located in the 5' or 3' region of the native gene, or within an intron.

A transgene expression construct contains one or more of the following elements: a 15 chicken lysozyme or ovalbumin promoter, a chicken lysozyme enhancer, and a matrix attachment region of a chicken lysozyme gene. The matrix attachment region (U.S. Patent No. 5,731,178, hereby incorporated by reference) preferably includes a region of the chicken 20 lysozyme gene spanning from position -11.7 to -8.8 or from position +5.3 to +9.0. One or both sequences are connected to the heavy and/or light chain of a tetrameric antibody to provide equal or equivalent expression of the two antibody subunits. Preferably, the region from -11.7 to -8.8 and/or +5.3 to +9.0 of the chicken lysozyme gene is attached to the 5' 25 and/or 3' end of a construct with a heavy and light chain antibody coding region each under the control of its own promoter. The invention includes a transgene expression cassette in which the heavy and light chain coding regions of an antibody are ligated together, each under the direction of its own promoter operably linked to a matrix attachment region. By "operably linked" is meant that a coding sequence and a regulatory sequence(s) are connected in such a way as to permit gene expression when the appropriate molecules (e.g., transcriptional 30 activator proteins) are bound to the regulatory sequence(s).

The invention also encompasses transgenic avian animals. The term "transgenic avian animal" refers to a member of an avian species (e.g., a chicken) that contains a transferred nucleic acid sequence, including a transferred protein-encoding and/or regulatory sequence,

such that the transferred sequence is integrated into a host chromosome. As a result of such transfer and integration, the transferred sequence may be transmitted through germ cells to the offspring of a transgenic chicken. Thus, transgenic chickens are created by introducing by a method of transfer, new nucleic acid sequences into germ cells.

5 A transgenic avian animal is any bird in which one or more, and preferably all of the cells of the animal, includes a transgene. The transgene is introduced into the cell, directly or indirectly by introduction into a precursor of the cell, e.g., a totipotent blastodermal cell of an avian egg, by way of deliberate genetic manipulation. The term genetic manipulation does not include classical cross-breeding, or *in vitro* fertilization, but rather is directed to the  
10 introduction of a recombinant DNA molecule into a blastodermal cell of an avian egg. This molecule may be integrated within a chromosome, or it may be extrachromosomally replicating DNA. Transgenic avians which include one or more transgenes encoding one or more polypeptides are within the scope of this invention. For example, a double or triple transgenic bird, which includes two or three transgenes can be produced.

15 A transgenic avian animal contains a nucleic acid sequence encoding a non-avian antibody polypeptide, e.g., a human antibody, in its genome. Other transgenic avian animals include those in which the genome has been engineered to contain a nucleic acid sequence encoding a non-avian insulin polypeptide such as a human or porcine insulin polypeptide or one which contains a nucleic acid encoding a calcitonin polypeptide, a growth hormone  
20 polypeptide, or a serum albumin polypeptide. Preferably, the polypeptides are of human origin. The transgenic avian animals express the transgenic nucleic acid, and the cells of the transgenic animal produce the transgenic polypeptides. Accordingly, the invention encompasses an isolated avian cell, e.g., an avian blastodermal cell, which contains a transgene encoding an antibody, an insulin, a growth hormone, a serum albumin, or a calcitonin  
25 polypeptide. The term "isolated" used in reference to a cell means that the cell is substantially free of other cell types or compositions with which it naturally occurs in a tissue of an animal.

The nucleic acids described herein are isolated. An isolated nucleic acid, e.g., an isolated gene, or a fragment thereof, to be transferred into a blastodermal cell of an egg is isolated by any of several methods well known to the art. For example, the nucleic acid molecules are recombinant and/or have been purified from the sequences which flank it in a naturally occurring state, i.e., a DNA has been removed from the sequences which are normally adjacent to the fragment, e.g., the sequences adjacent to the fragment in the genome

in which it naturally occurs. Thus, an isolated nucleic acid molecule is produced synthetically, or by treating mRNA derived from the transcription of the gene with a reverse transcriptase so as to produce a cDNA, or by the direct isolation of the nucleic acid from cells, bacterial clones, or from other sources.

5       The invention includes sequences which hybridize under stringent conditions, with all or part of the sequence reported in a reference sequence and retains a biological function of the reference nucleic acid. For example, the nucleic acid may contain one or more sequence modifications in relation to a reference sequence. Such modifications may be obtained by mutation, deletion and/or addition of one or more nucleotides compared to the reference  
10      sequence. Modifications are introduced to alter the activity of the regulatory sequence, e.g., to improve promoter activity, to suppress a transcription inhibiting region, to make a constitutive promoter regulatable or vice versa. Modification are also made to introduce a restriction site facilitating subsequent cloning steps, or to eliminate the sequences which are not essential to the transcriptional activity. Preferably, a modified sequence is at least 70% (more preferably at least 80%, more preferably at least 90%, more preferably at least 95%, more preferably at  
15      least 99%) identical to a reference sequence. In the case of a transcriptional regulatory element, the modifications do not substantially alter the transcription promoter function associated with the reference sequence (or a naturally-occurring lactoferrin promoter sequence). For example, promoter modifications are engineered to avoid the site of initiation  
20      of transcription and the TATA box. Modified transcriptional regulatory sequences such as enhancers retain at least 50%, preferably at least 75%, preferably at least 95%, and most preferably 100% of the enhancer activity of the reference sequence. Alternatively, the modified sequence directs a level of transcription that is greater than that of the reference sequence. For example, a modified enhancer directs at least 110% of the level of transcription  
25      associated with the reference enhancer sequence.

Nucleotide and amino acid comparisons are carried out using the Lasergene software package (DNASTAR, Inc., Madison, WI). The MegAlign module used was the Clustal V method (Higgins et al., 1989, CABIOS 5(2):151-153). The parameter used were gap penalty 10, gap length penalty 10.

30      Alternatively, the nucleic acids described herein hybridize at high stringency to a strand of DNA having the reference sequence, or the complement thereof and have transcription regulatory activity. Hybridization is carried out using standard techniques, such

as those described in Ausubel et al. (Current Protocols in Molecular Biology, John Wiley & Sons, 1989). "High stringency" refers to nucleic acid hybridization and wash conditions characterized by high temperature and low salt concentration, i.e., hybridization at 42 degrees C, and in 50% formamide; a first wash at 65 degrees C, 2X SSC, and 1% SDS; followed by a second wash at 65 degrees C and 0.1% x SSC. Lower stringency conditions suitable for detecting DNA sequences having about 50% sequence identity to a reference gene or sequence are detected by, for example, hybridization at 42 degrees C in the absence of formamide; a first wash at 42 degrees C, in 6X SSC, and 1% SDS; and a second wash at 50 degrees C, in 6X SSC, and 1% SDS.

Other features and advantages of the invention will be apparent from the description and the drawings and from the claims.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a diagram of a fertilized hen's egg.

Fig. 2 is a diagram of a transgene expression cassette.

#### DETAILED DESCRIPTION OF THE INVENTION

The avian reproductive system is distinct from mammalian reproductive systems in that the female can store sperm and fertilize a single ovum at a time. The new fertilized ovum is large, fragile, and filled with yolk as it enters the reproductive track. Early embryonic development occurs in the oviduct as the egg is formed around the ovum. As the ovum travels down the reproductive tract, it is surrounded by a protective layer of white albumen followed by an inner membrane and hardened shell before being laid (Fig. 1). At the time of oviposition, the ovum has matured from a single cell into a blastoderm (also known as the germinal disc) composed of 40-60,000 cells and development arrests until the hen has laid enough eggs and begins to roost. In the blastoderm state, all cells are totipotent and equally capable of contributing to the germ line of the developing chick.

Oviposition is the time at which the egg is laid. In the chicken, oviposition occurs at stage X (a freshly laid egg; about 20 hours of uterine age). The time at which a nucleic acid is introduced into the blastoderm or germinal disc is after oviposition but before incubation of the egg, i.e., before the first cell division after the egg is incubated. To effectively target blastodermal cells, DNA is therefore introduced into the blastoderm of an egg which has been incubated for 6 hours or less. Early stages of development of the egg are indicated by roman numerals, whereas stage of development of an embryonic chick are indicated by arabic

numerals. For example, stage X refers to a stage of development that occurs at about 20 hours uterine age and is characterized by oviposition, whereas stage 10 refers to a stage of embryonic chick development (characterized by tissue differentiation). No tissue differentiation has occurred in stage X of development. Accordingly, genetic manipulation occurs before differentiation of blastodermal cells into embryonic tissue.

The germinal disc is distinguished from the germinal crescent region in that the germinal disc contains undifferentiated blastodermal cells (at stage X or before), whereas the germinal crescent region appears in the early stages of chick embryo development (i.e., stages 3-5 or 9-11 of chick embryo development).

The cells of the blastoderm are genetically manipulated both *in vitro* or *in vivo* using gene delivery techniques and then used to produce transgenic or chimeric chickens by allowing development in the egg, transferring to a recipient unfertilized egg, or transferring to the testes of a sterile rooster for development into spermatogonia. The optimum time for transfection is between oviposition and several hours of activation of the egg in order to reduce the number of target cells for transfection.

#### Nucleic acid delivery and production of transgenic animals

The blastoderm is accessed by cutting or drilling a small hole in the egg shell (sitting upright) with a scalpel or drill and gently peeling back the inner membrane to expose the white albumen. The blastoderm automatically orients to the top of the yolk and is visualized under light. For the production of chimeric or transgenic chickens, the cells of the blastoderm are transfected *in vivo* by infusing DNA directly into the blastoderm using a syringe and small gauge needle. The DNA is naked or complexed with lipids or other suitable compounds to facilitate DNA uptake (e.g., DEAE-dextran). If the DNA is naked, the transfection efficiency is increased by passing an electrical current across the blastoderm or whole egg with a device such as a human heart defibrillator. If a current is passed across the whole egg, two additional holes are made in the egg shell to expose the inner membrane to the current since the shell will not conduct electricity.

Alternatively, the blastoderm is removed from the egg and pooled with the cells from several eggs using a small pipet. *In vitro*, DNA uptake by blastodermal cells is facilitated by such techniques as electroporation, DEAE-dextran treatment, calcium phosphate treatment, or lipofection. Following transfection, the cells are transferred into the germinal disc of an unfertilized egg for development of a transgenic chick or into the testes of a sterile rooster to

induce development in spermatogonia and sperm for breeding. Chicks produced are tested for the presence of the transgene according to known methods, e.g., by the polymerase chain reaction or southern blot analysis.

The overall efficiency of the nucleic acid delivery procedure depends on the method and timing of gene delivery. Transfection efficiency is optionally increase by methods such as 5 1) subjecting the blastoderm or cells derived from the blastoderm to several rounds of transfection. 2) adding a selectable marker such as but not limited to an antibiotic gene to the DNA vector and infusing the antibiotic into the yolk or testes following transfection or cell transfer.

10 The method is applicable to all birds including, but not limited to, chickens, turkeys ostriches, and geese. The critical factor for the efficiency of gene delivery is the timing of egg activation in relation to transfection. The optimal time to transfet the cells is after oviposition and within six hours of activation (post-incubation) so that the cells have started to grow but have not undergone a cell division.

15 The following are examples of the preferred embodiments of the inventions and specifically related to the production of transgenic chickens for the production of pharmaceuticals in eggs. The structure or composition of the transgene has little or no effect on the transfection efficiency of the methods described. Preferred transgene constructs include those carrying the ovalbumin promoter operatively linked to the human serum albumin gene, 20 human insulin gene, native or modified porcine insulin gene, calcitonin gene, or and gene encoding and antibody variable region. The invention includes transgenic chickens carrying any of the above mentioned genes directed to expression in the egg.

Example 1: In vivo nucleic acid delivery

Fertilized eggs are laid and collected (day 1). The eggs are shipped at room 25 temperature (unincubated) (day 2). Optionally, the eggs are allowed to sit upright for at least 24 hours (day 3) at room temperature (unincubated) prior to manipulation for nucleic acid delivery.

A fertilized hen's egg was collected and placed in a humidified incubator for between 1 and 6 hours to activate the egg. A hole was cut in the egg shell using a dremel and the inner 30 membrane was folded back. The egg was placed on a light to visualize the germinal disc which was infused with a lipid/DNA complex. The inner membrane was folded into place and the egg sealed with parafilm. The egg was placed back into the incubator and allowed to

develop to term. A blood sample or inner membrane sample was taken from the resulting chick and tested for the presence of the transgene by the polymerase chain reaction with transgene specific primers. A mosaic or chimeric chick is bred to a rooster to produce fully transgenic offspring.

5       Example 2: In vitro DNA delivery of blastodermal cells and transfer of transfected eggs to an unfertilized egg

Ten to twenty fertilized hen's eggs are placed in a humidified incubator for between 1 and 6 hours to activate cell division. A hole is cut in the egg shell using a dremel and the inner membrane folded back in order to visualize the blastoderm. The blastoderms are removed  
10      from the eggs with a large bore pipet tip and combine in a conical tube. The yolk is removed by washing with phosphate buffered saline, and the blastoderms collected by centrifugation. The cells are dispersed by treatment with collagenase or gentle pipetting and collect by centrifugation. The cells are resuspended in serum free media containing 1-5 g of transgene and allowed to sit at room temperature in an electroporation cuvette. The cells are gently  
15      resuspended and electroporated at 300-500V and 250-960uF. Following transfection, the cells are transferred into the germinal disc of an unfertilized egg by cutting a hole in the egg shell, folding back the inner membrane, and infusing the cells into the disc with a syringe and large gauge needle. The egg is sealed and incubated in a humidified incubator to allow development. A blood sample is taken from the resulting chick and tested for the presence of  
20      the transgene by the polymerase chain reaction with transgene specific primers using standard methods.

20       Example 3: In vitro DNA delivery of blastodermal cells and transfer of transfected eggs to rooster testes

Ten to twenty fertilized hen's eggs are placed in a humidified incubator for between 1 and 6 hours to activate cell division. A hole is cut in the egg shell using a dremel and the inner membrane folded back in order to visualize the blastoderm. The blastoderms are removed  
25      from the eggs with a large bore pipet tip and combined in a conical tube. The yolk is removed by washing with phosphate buffered saline, and the blastoderms collected by centrifugation. The cells are suspended in serum free media containing 1-5 g of transgene and allowed to sit at room temperature in an electroporation cuvette. The cells are gently resuspended and  
30      electroporated at 300-500V and 250-960uF. Following transfection, the cells are transferred

into the testes of a sterilized rooster to induce development into spermatogonia. The rooster is monitored for breeding and sperm collected to test for the presence of the transgene.

Example 4: Vector Construction

To target expression of insulin to the magnum cells of an egg laying hen, a genomic sequence encoding human insulin was operatively linked to a promoter which directs transcription of a nucleic acid to which it is operably linked in avian eggs. For example, the promoter is a human lactoferrin promoter. The construction of a human lactoferrin expression cassette is described in patent application USSN 09/490,801, the contents of which are hereby incorporated by reference.

For example, transcription regulatory elements are derived from a milk specific promoter, e.g., a mammalian lactoferrin gene promoter. The expression cassette contains a promoter region derived from the human lactoferrin gene operably linked to a heterologous sequence. A heterologous sequence is one that does not encode a lactoferrin polypeptide. The promoter region includes at least 20 nucleotides of the nucleotide sequence of SEQ ID NO:1. For example, the promoter region contains nucleotides 1-154 of SEQ ID NO:1 or 2.

Table 1: Human Lactoferrin promoter region

1	CTGGATCCTCAAGGAACAAGTAGACCTGGCCGCGGGAGT
41	GGGGAGGGAAAGGGGTGTCTATTGGGCAACAGGGCGGAAA
81	GCCCTGAATAAAGGGGCGCAGGGCAGGCGCAAGTGCAGAG
121	CCTTCGTTGCCAACGTGCCTCGAGACCGCAGACAT <b>GAAA</b>
161	GCATGTCTCCGCGGAAAA (SEQ ID NO:1)

BamH1 restriction site GGATCC (nucleotides 5-8) and Xhol site (nucleotides 140-145) are italicized. These restriction sites may be altered, e.g., replaced with other restriction sites or with nucleotides that do not represent restriction enzyme recognition sites.

Table 2: Human Lactoferrin promoter region

1	CTNNNNNTCAAGGAACAAGTAGACCTGGCCGCGGGAGT
41	GGGGAGGGAAAGGGGTGTCTATTGGGCAACAGGGCGGAAA
81	GCCCTGAATAAAGGGGCGCAGGGCAGGCGCAAGTGCAGAG
121	CCTTCGTTGCCAACGTGCNNNNNACCGCAGAC <b><u>ATGAAA</u></b>
	GCATGTCTCCGCGGAAAA (SEQ ID NO:2)

Optionally, the lactoferrin-derived promoter regions described above are linked to the nucleotide sequence of SEQ ID NO:3 (GENBANK™ accession no. S52659).

**Table 3: 5' Region of human lactoferrin gene**

1 cgaggatcat ggctcaactgc cacccttcata tcccaggcgc aaatggctt cccacttt  
61 ccicccaagt agctgggacc ataggcatac accaccatgc tgggctaatt ttgttatttt  
121 tttagagat gggggttcc ctatgaagcc caggctatgc ttgaactcc gggctcaagc  
181 gatcccccata tcctggcctc ccaaagtgct gggattacag gcatgagcca ctgtgcctcg  
241 cctagttact ctggcttaa gttcacatcc atacacacag gatattctt ctgaggcccc  
301 caatgtgtcc cacaggcacc atgctgtatg tgacactccc cttagagatgg atgttttagtt  
361 tgcctccaac tgatiaatgg catgcagtgg tgccctggaaa catttgtacc tgggtgtcg  
421 tgtgtcatgg gaatgttattt acggatgtta ttcttagaag cagtiattcta gcititgaat  
481 tttaaaatct gacatttatg gcgatgtta aaatgagggtt accatttcctt attgaataact  
541 atacaacacca aaaaagaaga aggaggagat ggagaaaaaaa aagacaaaaaa aaaaaaaaaagt  
601 ggttagggcat cttagccata gggcattttt ctcatggca aataagaaca tggaccacgc  
661 ctgggtggt ggcattccc ctctgaggc cctgtctgtt ttctgggagc tggattgtgg  
721 gtcicagcag ggcagggaga tacccatgg gcagctgccc tgagactctg ggcagcctct  
781 cttttcctg tcagctgtcc cttagctgtc gctgggggtg gtgggtcat ctttcaact  
841 ctcaagtcac tgctgagccca aggtgaaagc aaacccaccc gcccataactg gtccttaggc  
901 accctcaagg tcacitctg aagaagatag cagtcicaca ggtcaaggcg atctcaagt  
961 aaagaccctc tgctctgtt cctgcccctc agaaggcact gagaccagag ctgggacagg  
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1141 tggagtctg tccitccctca gggctttctg gagcctggat ctcaggaa caagtagacc  
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1261 tgaataaagg ggcgcaggc aggcccaagt ggcagagccct tcgttgc aagtcgcctcc  
1321 agaccgcaga catgaaaccc tgcctccctg tcctgctgtt ctcggggcc ctcggtgagt  
1381 gcagggtccctt gggggcgcga gcccctgtat gggcgtctcc tgcgcctgt ctgttaggc  
1441 ctgggtccc tggctccctg tggctggcg cggggctctc ggcgcggcgc gtcggcggc  
1501 ctacagccgg gaggcggccc ggacgcgggg ccagtcctt tcccacatgg ggaggaacag  
1561 gagctggctt cctcaagccg gatggggca cgcttagctc tgctcagac ttctcaaaag  
1621 gcctcccaagg cccctgtccc ttgtgtccctt gcctaaaggat ttggccccc ttgtattgt  
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1921 tggatggaaacc ctgtgtcaga gggtttggaga gggaaatgtc agccaaacag aaggaaggag  
1981 cagaaggaag gaaacaatgtc tcaatgtccat aacccaaatgtc atttctcggt tgctcagagg  
2041 gcactccccca ggcgtgcaca ttgtgtccat aatgcgtga gtgcgg (SEQ ID NO: 3)

The nucleic acid molecules and constructs described herein are isolated. By "isolated" is meant a nucleic acid molecule that is free of the genes which, in the naturally-occurring genome of the organism, flank the sequence of interest. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eucaryote; or which exists as a separate molecule (e.g., a cDNA or a genomic or cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences. It also includes a

recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequence. The term excludes large segments of genomic DNA, e.g., such as those present in cosmid clones, which contain a given DNA sequence flanked by one or more other genes which naturally flank it in a naturally-occurring genome.

5 Lactoferrin-derived transcription regulatory sequences, are attached to a nominal promoter (e.g., the nominal lactoferrin promoter or a heterologous promoter) which in turn is operably linked to a sequence to be transcribed. The heterologous sequence to be transcribed is a polypeptide-encoding sequence or antisense sequence. When incorporated into a  
10 transgenic mammal such as a member of an avian species, the regulatory sequences of the invention operably linked to a polypeptide-encoding sequence direct production of the encoded polypeptide in avian tissues. The lactoferrin-derived regulatory sequence, e.g., promoter sequence is positioned 5' to a heterologous nucleic acid sequence, e.g., a transgene, in a transcription unit. Portions of the lactoferrin-derived promoter region are tested for their ability to allow tissue-specific and elevated expression of a transgene using assays known in  
15 the art, e.g., standard reporter gene assays using luciferase, beta-galactosidase, or expression of an antibiotic resistance gene as a detectable marker for transcription.

All or part of one of the nucleotide sequences specified in a reference sequence, e.g., SEQ ID NO:1 or 2, its complementary strand or a variant thereof may be used in to direct transcription of a heterologous nucleic acid sequence such as a transgene in a transgenic  
20 mammal. A nucleic acid fragment is a portion of at least 20 continuous nucleotides identical to a portion of length equivalent to one of the reference nucleotide sequences or to its complement.

Other promoters which direct transcription in eggs of an avian animal are known in the art, e.g., the chicken ovalbumin promoter (GENBANK™ J00895 or M24999) and the chicken  
25 lysozyme promoter (GENBANK™ J00886 or V00429). An expression vector is constructed using a chicken ovalbumin promoter for expression of cloned sequences (Gannon et al., Organisation and sequences at the 5'end of a cloned complete ovalbumin gene. Nature 278:428434; Lai et al., The ovalbumin gene: Structural sequence in native chicken DNA are not contiguous. Proc. Natl. Acad. Sci. USA 75(5): 2205-2209; and Kaye et al., EMBO J. 3:1137-1144). Other regulatory elements which direct transcription of transgenes include a nuclear DNA attachment element which mediates elevated and position-independent gene activity (Stief, A., et al., Nature 341:343-345) and an attachment element for stimulation of

eucaryotic expression systems (Sippel et al., U.S. Patent No. 5,731,178). Other promoters useful to direct transcription of transgenes in eggs include the conalbumin, ovomucoid, and ovotransferrin promoters known in the art. For expression in blood or liver tissues, a promoter derived from the chicken beta globin gene is used (Foley et al., Proc. Natl Acad Sci USA 91:7252-7256).

Protein or polypeptide products to be expressed by the transgene include human insulin (GENBANK™ V00565), human calcitonin (GENBANK™ X15943; Broad et al., Nucl. Acids Res. 17:6999-7011), human serum albumin (GENBANK™ M12523, J04457), and a porcine single chain insulin. The nucleic acid sequence of the porcine single chain insulin sequence are shown below in Tables 4 and 5.

Table 4: Porcine Single Chain Insulin sequence 1

CTCGAGATGAAAAGATTGTTAACCAACACTTGTGCGGTTCCACTTGTGAAGCT  
TTGTACTTGGTTGCGGTGAAAGAGGTTCTTCTACACTCCTAAGGCTGCTAAGGG  
15 TATTGTCGAACAATGCTGTACCTCCATCTGCTCCTGTACCAATTGGAAAACACT  
GCAACTAGACTCGAG (SEQ ID NO:4)

Table 5: Porcine Single Chain Insulin sequence with lysozyme signal sequence

20 CTCGAGATGAGGGCTTGCTAACATCTTGGTGCTTGCTTCCTGCCCTGGCTGC  
TCTGGGGAAAAGATTGTTAACCAACACTTGTGCGGTTCCACTTGTGAAGCTT  
TGTACTTGGTTGCGGTGAAAGAGGTTCTTCTACACTCCTAAGGCTGCTAAGGGT  
ATTGTCGAACAATGCTGTACCTCCATCTGCTCCTGTACCAATTGGAAAACACTG  
25 CAACTAGACTCGAG (SEQ ID NO:5; bold type indicates signal sequence)

The invention includes sequences which hybridize under stringent conditions, with all or part of the sequence reported in a reference sequence and retains transcription regulatory function. For example, the nucleic acid may contain one or more sequence modifications in relation to a reference sequence. Such modifications may be obtained by mutation, deletion and/or addition of one or more nucleotides compared to the reference sequence. Modifications are introduced to alter the activity of the regulatory sequence, e.g., to improve promoter activity, to suppress a transcription inhibiting region, to make a constitutive promoter regulatable or vice versa. Modification are also made to introduce a restriction site facilitating subsequent cloning steps, or to eliminate the sequences which are not essential to the transcriptional activity. Preferably, a modified sequence is at least 70% (more preferably at least 80%, more preferably at least 90%, more preferably at least 95%, more preferably at least 99%) identical to a reference sequence. The modifications do not substantially alter the

transcription promoter function associated with the reference sequence (or a naturally-occurring lactoferrin promoter sequence). For example, modifications are engineered to avoid the site of initiation of transcription and the TATA box.

5       Example 5: Construction of a lactoferrin expression cassette

A lactoferrin expression cassette was constructed using lactoferrin transcription regulatory elements. The cassette contained 3Kb of promoter and 7Kb of 3' flanking sequence with unique SalI and NotI restriction sites at the 5' and 3'ends, respectively. In addition, the vector contained a unique XhoI site for the addition of heterologous coding sequences.

10      The human insulin gene was PCR amplified from human genomic DNA with the primers HNF3 (5'GCCCTCGAGGACAGGCTGCATCAGAA3'; (SEQ ID NO:6))/HINR3 (5'CTCGGTGCTCGAGGCGGCGGTGT3'; SEQ ID NO7) cloned into the vector pCR2.1 (Invitrogen, Carlsbad,CA) according to the manufacturer's instructions. The gene sequenced using the Amplicycle Sequencing kit (PE Applied Biosystems, Foster City, CA) to confirm that no base mutations had occurred during amplification. The gene was excised from the 15 vector pCR2.1 as and XhoI fragment and cloned into the XhoI site of the human lactoferrin expression cassette. The orientation of the insulin gene was confirmed by restriction analysis and DNA sequencing. The completed vector was designated HL31 and could be excised from the bacterial backbone as a SalI to NotI fragment.

20       Example 6: Preparation of HL31 for Transfection

To remove bacterial sequences from the vector HL31, it was digested with SalI and NotI to completion, extracted with phenol/chloroform, chloroform, and fractionated on a 1% Tris Acetate agarose gel. The transgene was excised from the gel and electro-eluted in dialysis tubing containing 1 x Tris-Acetate buffer. The eluted DNA was transferred into an eppendorf tube and precipitated by adding 1/10th volume 3M sodium acetate pH5.2, 2.2 volumes ethanol, and incubating at -20 degrees C for 24 hrs. The DNA was collected by centrifugation at 25 13,000 x g for 10 minutes and resuspended in distilled sterile water. The concentration of DNA was estimated by Tris-borate gel electrophoresis against a known quantity of lambda HindIII digested standard. The DNA was stored at 4 degrees C.

30       Example 7: Production of Chimeric Transgenic Chickens

Developmental stage X chicken eggs from white leghorn chickens were obtained from a commercial vendor (Charles River/SPAFAS, North Franklin, CT). The eggs were allowed

to acclimate for 24 hrs at room temperature prior to transfection to allow the germinal disc/blastoderm to orient to the top of the egg. Using a dremel, a 1-2cm circular cut was made in eggshell over the air sac region. Before proceeding, the transgene was prepared for injection at the following concentrations; DNA 5-15 ng/ l, 10-15% phospholipid (Life Technologies, Grand Island, NY), 50% Dulbecco's Modified Eagles media, and the remaining volume with phosphate buffered saline. The DNA/lipid is allowed to stand at room temperature for a minimum of 15minutes prior to injection into the egg.

Just prior to injection, the eggshell was removed to expose the inner shell membrane and allow the visualization of the germinal disc. If the germinal disc could not be visualized a small opening was made in the membrane. Following location of the germinal disc, the DNA/lipid solution was injected directly into the center of the disc. Nucleic acid solutions (construct encoding human insulin) were injected in a volume of 50-100 l with a 1cc syringe and 27 gauge needle or in a volume of 10-20 l with a microinjection needle attached to a Hamilton syringe. Table 6 shows data from representative experiments. 228 eggs were injected and 26 chicks were hatched (Table 6)

Table 6: Summary of Egg Injection Experiments

Experiment	Conc. Of DNA (ng/ l)	Percent Phospholipid	# of Eggs Injected	# of Chicks Hatched
32	5	15	16	3
33	10	15	22	2
34	5	20	22	9
34a	7.5	10	22	2
35	15	10	21	1
36	15	10	21	3
37	10	5	30	4
38	5	10	30	2
39	10	10	30	0
40	10	10	30	0

Chimerism of the hatched chicks was tested by PCR analysis of genomic DNA isolated from the inner shell membrane (post-hatching), or the liver, kidney, and reproductive organs upon necropsy (Table 7). Out of the 3 chicks tested in experiment 32, 2 showed a strong PCR signal in the inner shell membrane sample. Upon maturation, these chicks are bred to confirm germline transmission of the transgene. PCR analysis of necropsy samples (membrane and reproductive organs) taken from 4 chicks derived from experiments 32 & 33 revealed that 1 out of 4 chicks had detectable levels of the transgene in its reproductive organs.

Table 7 shows results from a representative analysis of tissue expression of human insulin transgene in chimeric chickens produced as described above.

Table 7: PCR analysis of Genomic DNA Isolated from Chimeric Chicks

Experiment	Chick	Tissue	Sample	Status
		Inner Membrane	Reproductive Organ	
32	C	NEG.	Not tested	Alive
	E	POS.	Not tested	Alive
	M	POS.	Not tested	Alive
	N	NEG.	NEG.	Dead
	O	POS.	NEG.	Dead
33	B	Not tested	Not tested	Alive
	BB	POS.	POS.	Dead
	FF	POS.	NEG.	Dead
34	AA	NEG.	Not tested	Alive
	CC	POS.	Not tested	Alive
	EE	NEG.	Not tested	Alive
	II	NEG.	Not tested	Alive
	KK	NEG.	Not tested	Alive
	QQ	NEG.	Not tested	Alive
	VV	POS.	Not tested	Alive
	ZZ	POS.	Not tested	Alive
	AAA	POS.	Not tested	Alive
	QQQ	POS.	Not tested	Alive

Marker transgenes such as MT-beta-Gal, and CMV-GFP were also transfected into blastodermal cells *in vivo*. Chicks were produced and tested for tissue expression of the transgene as described above. The transgenic DNA was detected in the following tissues which were tested: heart, liver, and kidney.

These data indicate that the methods and constructs described herein are useful to transfect avian blastodermal cells and to make transgenic avian animals which produce a desired transgenic polypeptide.

Example 8: Method for the production of tetrameric antibodies

Unlike many prior art methods for production of transgenic chickens, the methods of the invention do not utilize retroviral particles. Many of those methods use non-replicating retroviruses, which contain a gene of interest, to infect a developing egg. This process has several limitations which include a restriction on the size of the gene packaged in the viral coat, and instability of expression from retroviral vectors. In addition, retroviral gene delivery is severely limited in its ability to produce high levels of multimeric proteins since each coding region would optimally require its own promoter. To circumvent this problem, some researchers have proposed the use of IRES sequences which allow for bicistronic mRNAs; however, the expression from these types of constructs is generally low unless a selection marker is used.

To overcome these drawbacks, a technology was developed which employs *in vitro* gene delivery methods to the creation of transgenic chimeric chickens. This method has the advantage of being able to deliver a transgene of up to 100Kb and allows for high level production of multimeric proteins such as antibodies. The flexibility in vector design allows the construction of transgene similar to those used in the mammary gland expression system which employ full length promoters, genomic coding regions, and 3' flanking regions. The method described herein utilizes the full 8Kb ovalbumin promoter, genomic or cDNA coding regions, and 3' flanking sequence. In addition, high level production of multimeric proteins is made possible because expression of each subunit is directed by its own promoter. The transgenes are transfected individually or ligated together to ensure co-integration which greatly facilitates germline transmission of all subunits (Step 1 and 2 of Fig. 2).

To ensure the equal expression of both subunits of the antibody, the transgene is insulated from the effects of the surrounding chromatin structure by including elements such as matrix attachment regions (Step 3 of Fig. 2). Unlike prior art methods which have utilized matrix attachment region, the constructs described herein result in equal expression of multimeric proteins. The elements are called A-elements, MAR for matrix attachment regions, SAR, scaffolding attachment regions, DCR for dominant control region, and insulators.

Other features, objects, and advantages of the invention will be apparent from the description and from the claims. In the specification and the appended claims, the singular forms include plural references unless the context clearly dictates otherwise. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly

understood by one of ordinary skill in the art to which this invention belongs. All patents and publications cited in this specification are incorporated by reference.

Other embodiments are within the following claims.

What is claimed is:

1. A method of introducing a nucleic acid molecule into the genome of an avian species, comprising contacting *in vivo* a blastodermal cell of a fertilized egg with said nucleic acid molecule, wherein said nucleic acid molecule is not associated with a viral coat protein and wherein said nucleic acid molecule is introduced directly into the germinal disc of said egg in a volume of greater than 1 microliter and less than 0.5 milliliters.
2. The method of claim 1, wherein said volume is 5-100 microliters.
- 10 3. The method of claim 1, wherein said volume is 40-60 microliters.
4. The method of claim 1, wherein said volume is 10-20 microliters.
- 15 5. The method of claim 1, wherein said nucleic acid molecule is introduced into said egg in a volume that is less than the volume of the germinal disc.
6. The method of claim 1, further comprising exposing said cell to an electrical current *in vivo*.
- 20 7. The method of claim 1, wherein said avian species is selected from the group consisting of a chickens, an ostrich, an emu, a turkeys, a duck, a goose, a quail a parrot, a parakeet, a cockatoo, and a cockatiel.
8. The method of claim 1, wherein said avian species is a chicken.
- 25 9. The method of claim 8, wherein the breed of said chicken is selected from the group consisting of White Leghorn, White Plymouth Rock, Barred Plymouth Rock, Rhode Island Red, New Hampshire and Dark Cornish.
- 30 10. The method of claim 1, wherein said blastodermal cell is contacted with said nucleic acid during developmental stage X of said egg.

11. The method of claim 1, wherein said blastodermal cell is contacted with said nucleic acid at a time after oviposition but before incubation of said egg.

5 12. The method of claim 1, wherein said nucleic acid comprises a sequence encoding an antibody or fragment thereof.

13. The method of claim 1, wherein said antibody or fragment thereof is human.

10 14. The method of claim 1, wherein said nucleic acid comprises a sequence encoding an insulin polypeptide, a growth hormone polypeptide, a calcitonin polypeptide, or a serum albumin polypeptide.

15 15. The method of claim 14, wherein said insulin polypeptide is a porcine single chain insulin polypeptide.

16. The method of claim 1, wherein said nucleic acid comprises a egg-specific transcriptional regulatory element.

20 17. The method of claim 16 wherein said regulatory element comprises a matrix attachment region of a chicken lysozyme gene.

18. The method of claim 17, wherein said region comprises a sequence from position -11.7 to -8.8 or from position +5.3 to +9.0 of the chicken lysozyme gene.

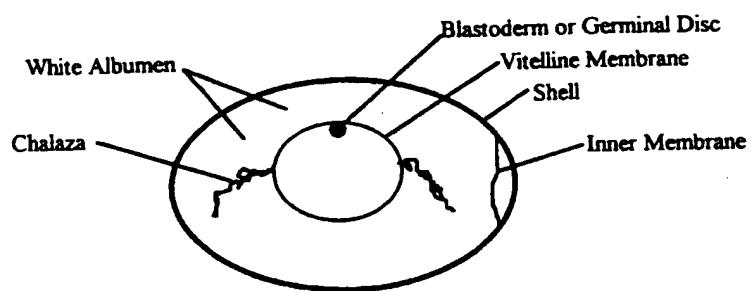
25 19. The method of claim 17, wherein said region is operably linked to a sequence encoding an antibody chain or fragment thereof.

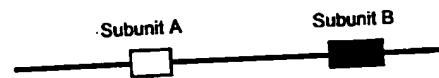
20. The method of claim 17, wherein said region is operably linked to a first sequence encoding an antibody light chain and a second sequence encoding an antibody heavy chain.

30 21. A transgenic avian animal, the genome of which comprises a nucleic acid sequence encoding a non-avian antibody polypeptide.

22. The animal of claim 21, wherein said sequence encodes a human antibody polypeptide.
- 5           23. A transgenic avian animal, the genome of which comprises a nucleic acid sequence encoding a non-avian insulin polypeptide.
- 10          24. A transgenic avian animal, the genome of which comprises a nucleic acid encoding a non-avian polypeptide, wherein said polypeptide is selected from the group consisting of a calcitonin polypeptide, a growth hormone polypeptide, and a serum albumin polypeptide.
25. An isolated avian blastodermal cell comprising a transgene encoding an antibody, an insulin, a growth hormone, a serum albumin, or a calcitonin polypeptide.

Figure 1



**Figure 2****1.****2.****3.**